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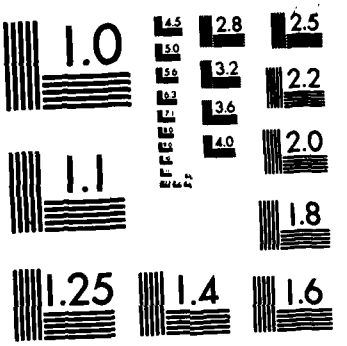

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During the ~~first year of a three year~~ research project to investigate the sublethal effects of the water soluble fraction (WSF) of JP-4, a constant flow water soluble fractionator for the JP-4 was constructed. Procedures for chemical analyses to determine the percent of the WSF were developed and used. Static and dynamic bioassays were performed using the bluegill, Lepomis macrochirus. Blood chemistry tests were performed on control and exposed fish. Electron micrographs were taken of gill and liver tissue from control and exposed fish. Equipment and protocols were developed for measurement of respiration rates and preference/avoidance behavior of fish exposed to sublethal concentrations of the WSF of JP-4

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AFOSR-TR- 83-0443

Sublethal Effects of JP-4 on Lepomis Macroch

I. INTRODUCTION

A. This is the annual report of the first year of grant AFOSR-82-0059, "A novel approach for predicting sublethal effects of toxicants to aquatic organisms". Funding for this first year of the three-year program had an offical starting date of 1 November 1981, but initial funding was not actually received until 1 February 1982.

B. In spite of the late starting date, most intended first-year goals were met. This was due to the fact that grant approval was received and the University Center for Environmental Studies (UCES) at Virginia Polytechnic Institute and State University (VPI&SU) was able to cover initial expenses.

II. RESEARCH OBJECTIVES

A. The initial research objectives as outlined in the grant proposal are:

1. Determine the acute and chronic toxicity of jet fuel to the freshwater bluegill Lepomis macrochirus after conventional and episodic dosing.

2. Determine the effects of sublethal concentrations of jet fuel water soluble fractions on fish breathing behavior.

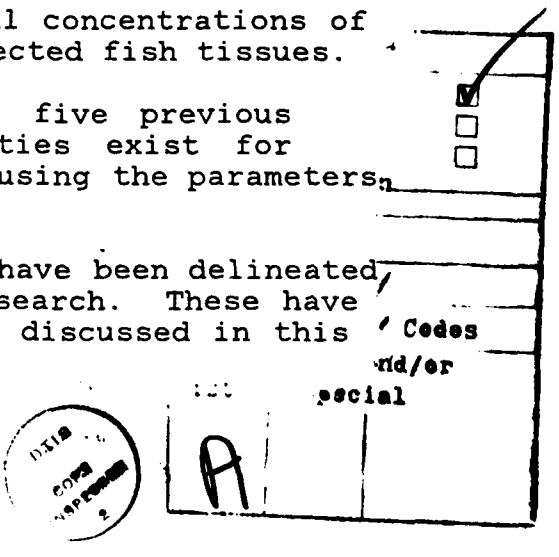
3. Determine the effects of sublethal concentrations of jet fuel water soluble fractions on fish swimming behavior.

4. Determine the effects of sublethal concentrations of jet fuel water soluble fractions on fish blood chemistry.

5. Determine the effects of sublethal concentrations of jet fuel water soluble fractions on selected fish tissues.

6. Compare data collected for the five previous objectives and determine if capabilities exist for predicting sublethal effects of stress using the parameters measured.

B. Some additional research objectives have been delineated for the second year of the proposed research. These have not been initiated yet and will not be discussed in this report.



III. PROGRESS TO DATE

A. Overview

1. The project is progressing well. Most of the first

year goals have been satisfied in spite of the delay in receiving operating funds.

2. A few modifications have been made in the research scheduling, primarily due to the availability of personnel with specific interests and abilities. Due to Mr Coiner's interest in doing an independent study as part of his requirements for a B.S. degree and his background in biochemistry, collection of background data on blood parameters of the bluegill was started during this first year of research rather than during the final year as had originally been planned. Another change is that much of the equipment has been updated and/or modified as well as tested during this first year. This resulted in a larger undertaking than originally planned for the preference avoidance equipment and involved many manhours and funds that were not planned for this area. This will be discussed in more detail below.

B. Jet Fuel

1. The jet fuel that is used throughout this research was obtained from AFWAL/POSF Wright-Patterson AFB OH. A quantity of 110 gallons of petroleum derived JP-4 (PJP-4) and 110 gallons of shale derived JP-4 (SDJP-4) is on hand. Another 55 gallons of the SDJP-4 is on hold at AFWAL/POSF in case it is needed later in the research.

2. Both these jet fuels have been completely chemically characterized by AFWAL/POSF so no further characterization is contemplated as part of this research. However, the analytical procedures have been developed that will be used to determine the concentrations of jet fuels in the water soluble fractions (WSF) to which the fish will be exposed during this research. A Varion 3700 Gas Chromatograph with a 6 foot column of 3% SE30 is being used. This procedure is similiar to the one used at AMRL/THE Wright-Patterson AFB OH in its investigations into the toxicity of different jet fuels.

3. Over 200 samples have been analyzed to date in support of bioassays with the WSF of jet fuels.

4. Procedure

a. The procedure for concentrating the samples of the WSF of jet fuels is one that was modified from a USEPA procedure. Samples of 100 ml are collected in a volumetric flask and then 1 ml of hexadecane is added. The sample is mixed for 3 minutes and is then allowed to settle for at least another 3 minutes. The hexadecane layer is removed and stored in a small glass vial with a teflon lid.

b. Settings on the gas chromatograph are 20 ml/min of nitrogen as the carrier gas, 30 ml/min of hydrogen and 300 ml/min of air using a flame ionization detector. The oven

of the gas chromatograph is programmed to maintain 40° for the first 5 minutes, and then it rises by 10° per minute till a final temperature of 200° is reached. This final temperature is held for 5 minutes, and then the gas chromatograph recycles for another analysis. A sample of 6 ml is usually injected for each analysis, and analyses are replicated at least twice.

c. Chromatograph standards are prepared by mixing benzene, napthalene, toluene, ortho-, meta-, and para-xylene into methylene. This is the same procedure used by AMRL/THE.

d. Standards for determining the percentage of the WSF are prepared by mixing a 5% jet fuel/water mixture for 3 hours and then settling for another 3 hours. The aqueous layer is then removed and treated as a normal sample. This mixture is considered to be a 100%, or saturated, WSF.

C. Modification of Equipment

1. A constant flow device for delivering the WSF of the JP-4 to the various testing equipment has been designed and built. It has been successfully used for dynamic bioassays and in dosing fish for later blood chemistry and electron microscopy. Figure 1 is a schematic of the design of the WSF device.

2. Due to the volatility of the WSF of JP-4, modifications were made to the existing all-glass Sprague-Brungs dilutor. These modifications include replacement of head boxes with smaller ones with glass lids, replacement of the mixing chambers with glass tubing and Y-connectors, and enclosing or sealing all other possible points where volatilization might occur. Several successful dynamic 96-hour bioassays have been performed using this equipment.

3. The respiration rate monitoring equipment that had been used for previous studies at UCES has been modified to run tests with the WSF of the jet fuels. Respiration data are collected using a PDP-8 computer whose performance is questionable at this time. It was repaired once this year, using UCES funds, but it still is sporadic in its operation. A decision will be made early in the second year of research whether to continue with the PDP-8 or to rewrite the data collection program for the other in-house computer system, a Cromemco Z-2. A rewrite of the entire program would be very time consuming and would require the services of an experienced programmer. However, this may prove more cost effective if the PDP-8 cannot be brought to a more reliable stage of operation.

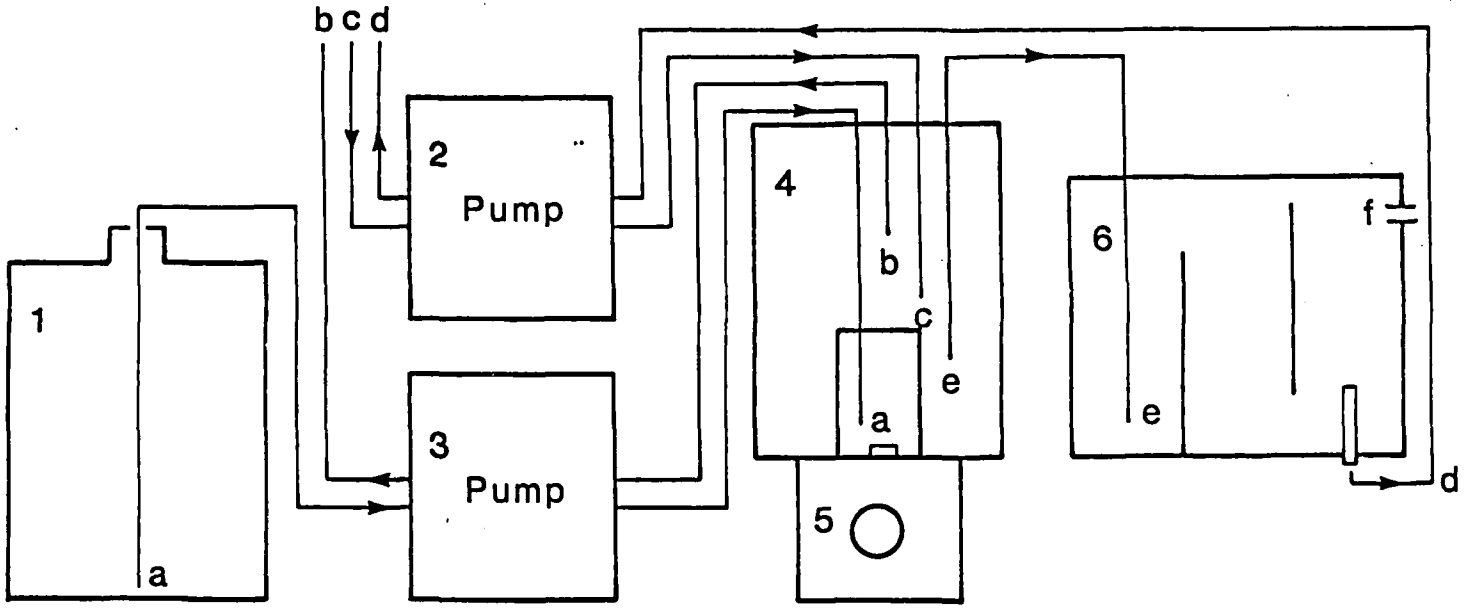


Fig. 1: Schematic of JP-4 Water Soluble Fractionating Device.

Legend: Jet fuel is pumped (line a via pump #3) from reservoir (#1) to mixing chamber (#4) where it is mixed by a magnetic stirrer (#5) with carbon filtered tap water (c via #2). The water soluble fraction of the jet fuel is siphoned (e) from near the bottom of mixing chamber (#4) into a baffled settling chamber (#6). The settled WSF is pumped (d via #2) from the settling chamber to the experimental equipment (bioassay dilutor system, respiration monitoring equipment or preference/avoidance chamber). Excess fuel is removed from the mixing chamber (b via #3) and overflow from the settling chamber goes to the drain (f).

4. The preference/avoidance monitoring equipment had not been used for a considerable period of time and needed repair. The computer program that collects data for this system was rewritten and a new piece of hardware has been designed and constructed. This new program is an updated version of the older system and is capable of monitoring more then one fish at a time. It is currently operational and will be used to generate data in the second year of the research.

5. The Gilford 3500 automated blood analyzer, that is on loan to the UCES from the U.S. Army, had been used very recently and is in very good working order. It has been used to collect data on the background or "normal" levels of certain blood parameters during this last year.

D. Results

1. As mentioned above, the jet fuels being used in this study have been completely chemically characterized by AFWAL/POSF so no further chemical characterization is planned. Analytical procedures have been validated by comparing present results with those from other USAF laboratories. Sufficient accuracy and reproducibility have been obtained which allows determination of the concentration of the WSF of the jet fuels to which the fish are being exposed.

2. Bioassays:

a. Static bioassays were performed initially with both the SDJP-4 and the PJP-4. Due the volatility of the fuel mixtures, these bioassays are essentially only 24-hour tests even though they were carried out for a full 96 hours. No further deaths occurred in any of the tests after the first 24 hours. The neat tests (jet fuel layed on the water and gently mixed in) are reported as ppm volume/volume concentrations. The WSF results are reported as percent WSF as compared to test solutions where 5% jet fuel was mixed for 3 hours and then allowed to settle for the same amount of time. (This is similiar to the technique used at AMRL/THE.)

1) Neat PJP-4: 24-hour LC50 = 1.8ppm
2) Neat SDJP-4: 24-hour LC50 = 5.6ppm
3) WSF PJP-4: 24-hour LC50 = 55%WSF
4) WSF SDJP-4: 24-hour LC50 = 70%WSF

b. Only dynamic bioassays have been performed on the PJP-4 to date. Three complete 96-hour bioassays were performed and resulted in an average LC50 of 26.6% WSF.

3. Test runs have been performed on the respiration equipment; however, no actual runs were made where fish were exposed to WSF jet fuel. The associated toxicant dosing

equipment was used to dose fish for later analysis of blood and tissues.

4. No full scale runs of the preference/avoidance equipment have been performed. This will require some modification of the WSF delivery system so that a sufficient flow is achieved. This will be worked on during the second year of research.

5. Over 100 samples of blood from the bluegill have been analyzed to date with the Gilford 3500 automated blood analyzer. Several manual tests have also been performed on each blood sample as well. Two different populations were tested. The first group was obtained from a fish hatchery in PA, as this hatchery is the source of fish for all other tests, i.e., bioassays, respiration and preference/avoidance. However, the expense of shipping the large fish needed for blood analysis became prohibitive. Therefore, fish were captured from a small pond located on the grounds of the Veterans Administration (VA) Hospital in Salem, VA. Approximately 50 samples of blood from each population have been analyzed to date.

a. The technique used to obtain blood is to anesthetize the fish with a 5% benzocaine solution. The tail is then excised and as much blood as possible is collected using 1 ml needleless syringes. Since one fish cannot provide sufficient blood for the entire battery of tests, blood from two fish is pooled. The syringes are either coated with heparin or not, depending on which parameters the blood is to be analyzed for. Heparin interferes with certain of the procedures used with the Gilford 3500. The heparanized blood samples are then centrifuged for 10 minutes at 2,000 rpm and placed on ice. The nonheparanized samples are allowed to clot after which all the blood samples are centrifuged at 13,500 rpm. The analyses are performed within the time frame outlined in the Gilford 3500 procedures manual. The other blood analyses, such as hematocrit, hemoglobin and red blood cell counts, are performed according to accepted procedures.

b. Results of the fish blood analyses from the pond at the VA Hospital are found in Table 1.

c. Only a few analyses have been performed on blood taken from fish that were exposed to the WSF of jet fuel. Insufficient number of analyses prohibits statistical comparisons with results for unexposed fish. Results to date are in Table 2.

Table 1: Levels of selected ions, enzymes, and other blood parameters for the bluegill.

Parameter (units)	N	Mean	Stand.Dev.
Hematocrit (%)	43	34.55	4.52
LDH (U/l)	39	2,027.41	1,062.0
GOT (U/l)	27	460.35	133.20
Total Protein (g/dl)	41	3.95	0.95
Inorganic Protein (mg/dl)	34	30.24	14.02
Albumin (g/dl)	37	1.49	0.25
Chloride (mg/dl)	33	169.09	94.03
Calcium (mg/dl)	36	17.21	4.52
Magnesium (mg/dl)	34	3.89	1.88

Note: Tests performed on a Gilford 3500 automated blood analyzer were done using reagents and procedures that have been proven to be accurate for the analysis of human blood parameters. It is not correct to say that these values are the true values for the bluegill. Rather, they are values that are approximated by these procedures.

Table 2: Mean levels of selected ions, enzymes, and other blood parameters for bluegill exposed to 8% WSFPJP-4.

Parameter (units)	15 hr	24 hr	48 hr
Hematocrit (%)	-	-	51
LDH (U/l)	2,145.0	1,865.5	4,926.8
GOT (U/l)	282.1	340.4	459.4
Total Protein (g/dl)	6.8	6.7	5.9
Inorganic Protein (mg/dl)	25.9	26.8	18.7
Albumin (g/dl)	2.1	2.2	2.0
Chloride (mg/dl)	90.0	89.7	54.6
Magnesium (mg/dl)	7.0	-	4.8

Note: Tests performed on a Gilford 3500 automated blood analyzer were done using reagents and procedures that have been proven to be accurate for the analysis of human blood parameters. It is not correct to say that these values are the true values for the bluegill. Rather, they are values that are approximated by these procedures.

d. Some trends are appearing in the blood analysis from the treated fish. There are some changes occurring in the levels of liver enzymes, as well as some differences in the levels of some ions. Too little data is available yet to make any definitive statements. In addition, exposure times have been short and at low dosage.

6. Several samples of gill and liver tissue have been taken from both control fish and fish exposed to WSFSDJP-4. These samples have been examined under a Transmission Electron Microscope (TEM).

a. Samples were prefixed in glutaraldehyde for 2-3 hours and then postfixed in osmium tetroxide for a similiar time period. They were then dehydrated with a series of increasing concentrations of ethanol. The samples were mounted in Spurr's resin and thinsliced on an ultramicrotome.

b. Gill tissue was also fixed using a mixture of pre- and post-fixatives containing ruthenium red dye to examine the membranous areas more closely.

c. Although only a few samples have been done to date, a clearly discernable difference occurs in the integrity of both types of tissue after exposure to the WSF of the jet fuel.

IV. PERSONNEL

A. Dr John Cairns, Jr., University Distinguished Professor and Director of the UCES, and Dr Arthur L. Buikema, Jr., Professor of Zoology and Assistant Director of the UCES, serve as Principal Investigators for this project.

B. The primary researcher on this project is Capt Thomas R. Doane, a graduate student working on a PhD in Zoology in the Department of Biology. He started at VPI&SU in July 1981 and has been working on this project since that time. His education is being paid for by the Air Force Institute of Technology (AFIT). He does not receive any financial assistance from this grant.

C. Hiring/Training of Research Personnel

1. Mrs Sylvia Sanford was hired on 1 Mar 82 as the primary technician on this project. She had been an employee of UCES for over a year and had been working on other UCES projects during that time period.

2. Mr Brewer Pedin has been hired on an hourly basis to work as a electronic technician and computer programmer. He is a senior at VPI&SU and has worked on similiar projects at UCES in previous years. He will be leaving the project as a regular employee soon after completion of this first year of the research. He may be hired on an occasional hourly basis

in the event of a failure in any of the electronic devices used in this research.

3. Mr Leonard Coiner, a senior in Biochemistry at VPI&SU, performed an independent study under Dr Buikema's direction. He helped establish the baseline data on blood chemistry of the bluegill Lepomis macrochirus. Mr Coiner did not receive any financial assistance from the grant.

4. Mr Douglas Bloem, a former graduate student working on a MS at UCES, was supported with a partial research assistantship during winter quarter. He did his researc· ɔn some of the equipment that will be used in this project d was training the new personnel, specifically Capt Doane .d Mrs Sanford, on the use of that equipment.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